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Regulation of Superoxide Anion Radical–Superoxide Dismutase System in the Avian Thyroid by TSH with Reference to Thyroid Hormonogenesis

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This study shows that superoxide dismutase is present in the thyroid gland of pigeons as a constitutive enzyme serving as an antioxidant against oxygen toxicity. Exogenous administration of thyrotropin induced thyroidal superoxide dismutase with a simultaneous burst in superoxide anion radical levels during the initial phase of hormone treatment. The superoxide radical generated was completely scavenged by SOD during the late phase of TSH-treatment, presumably as an adaptive measure to check the oxygen burst. TSH failed to augment serum T_3 levels, although the thyroxine level in the serum was elevated. The peak level of SOD activity profile in the thyroid gland correlated very well with the peak level of thyroxine concentrations in the serum of pigeon. It is reasonable to postulate that the thyroidal SOD in homeotherms serves a dual role, firstly as a strategic antioxidant enzyme to protect the thyroid gland against the degenerative influence of toxic oxyradicals and secondly to provide H_2O_2 for thyroid hormone biosynthesis. Our results confirm the previous observations that TSH is mainly thyrotropic in birds and that it has no influence on the peripheral activation of thyroxine to triiodothyronine by stimulating the extra thyroidal 5′-deiodinase activity.

Numerous studies have demonstrated the presence of superoxide anion radical ($O_2^{\cdot-}$) and superoxide dismutase in the thyroid gland of mammals and birds (1-3). In addition to the antioxidant and protective role of SOD against oxygen toxicity, it is believed that the dismutation of $O_2^{\cdot-}$ into H_2O_2 by SOD in the thyroid serves as an alternate H_2O_2-generating machinery to drive the H_2O_2-dependent and peroxidase-mediated thyroid hormonogenesis (1). Verma et al. (1) suggested that in addition to being a substrate for H_2O_2 generation, $O_2^{\cdot-}$ mediates the activation of iodine into an active free radical form ($I^{\cdot-}$), which could be the intermediate that is incorporated onto tyrosine.

Thyroid stimulating hormone (TSH) stimulates proliferation, differentiation and metabolism of thyroid follicle cells (5, 6). TSH is known to stimulate cAMP dependent DNA synthesis and associated differentiation in follicular cells (5-11). Data have accumulated concerning the effect of TSH on specific chemical and enzymatic events in the thyroid (5-8, 12-21). Out of the thyroid hormone synthesizing enzymes, iodide peroxidase is found to be augmented by chronic administration of TSH regimens (22, 23). TSH depletion by hypophysectomy induced a decrease in the enzyme activity, which could be reversed by TSH administration and these changes in the enzyme activity required only a short interval (24). It has also been shown that the modulation of thyroidal iodide peroxidase by TSH is possibly through its effects on the biosynthesis of this protein (25). TSH also regulates the expression of immediate early genes including c-fos and jun-B (6). These immediate early genes encode nuclear transcription factors capable of modulating gene expression and ultimately cell growth, differentiation and metabolism (6, 9).

The established thyrotrophic function of TSH and the growing importance of $O_2^{\cdot-}$/SOD system in H_2O_2-dependent thyroid hormone biosynthesis promoted us to investigate the possible regulation of thyroidal $O_2^{\cdot-}$/SOD system by TSH and its correlation with serum T_3 and T_4 levels in birds. Uptill now, this aspect of TSH interaction with the thyroid gland is unresolved. In the present investigation, changes in the levels of $O_2^{\cdot-}$, SOD, thyroxine (T_4) and triiodo thyronine (T_3) in a 3 hr period of TSH stimulation of the thyroid in pigeons were monitored to understand any relation between the $O_2^{\cdot-}$/SOD system and thyroid hormogenesis.

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MATERIALS AND METHODS

Reagents. Bovine thyroid stimulating hormone, thyroxine, triiodothyronine, trizma base, trizma HCL, Triton X-100, diethyl dithio carbamic acid sodium salt and N-2-hydroxy ethyl piperazine-N′-2-ethane sulfonic acid were obtained from Sigma Chemical Co., USA. N-t-butyl-α-phenyl nitrone was obtained from Aldrich Chemical Co., Milwaukee, WI. Pyrogallol was obtained from Loba Chemie, India and ethylene diamine tetra acetic acid from BDH, India.

Animals. Adult male blue rock pigeons (*Columbia livia intermedia*) weighing around 250 gms were purchased from a local bird supplier. Birds were maintained and acclimatized in a temperature controlled (27±1°C) room at light:dark regimen of 14hr:10hr for 15 days before they used for the experiments. During the period of acclimatization, food and water were provided ad libitum.

Methods. Seven groups of adult male birds, each with six healthy individuals were established in separate wire net cages (50×40×30 inches). Birds of six groups received a single subcutaneous shot of 20μg TSH in 1 ml physiological saline. Control birds received equivalent amount of physiological saline alone. The TSH injected birds were sacrificed by cervical dislocation at different time periods after TSH injection, viz., 20, 40, 60, 80, 120 and 180 minutes. The thyroid gland was excised, cleared of adhering fat and washed several times with chilled physiological saline to remove blood. The glands were weighed and then processed for SOD assay and free radical quantification. A minimum of three replicates were taken for each analysis.

Before sacrificing the animals, approximately 2 ml blood was drawn from the branchial vein of each bird. Blood samples were maintained for 4 hr at room temperature to allow clot formation and retraction and then refrigerated at 4°C overnight. Samples were centrifuged and sera were removed and stored frozen at −20°C for subsequent radioimmunoassay of T_4 and T_3.

Thyroxine (T_4) and Triiodothyronine (T_3) assay. Serum concentration of T_4 and T_3 were measured following modified method of Brown *et al.* (26) using RIA kits for Bhabha Atomic Research Center, Bombay, India. Standards were made up in pigeon's free to T_4 and T_3.

Spin trapping of superoxide radical generated by the thyroid gland. Preweighed thyroid glands were homogenized in HBSS (27) adjusted to pH-7.2 using 50 mM HEPES, at 2500 rpm (2 cycles, 10 seconds each) using a Polytron homogenizer equipped with PT-10 accessory, so as to disperse the follicle cells. The extract was incubated with 50 mM PBN and 1×10^{-3}M DDC (final concentration) for 1 hour at 27°C. After incubation, 25 μl aliquots were transferred to glass capillaries (Clinicon International, GmbH) and one end flame sealed taking care not to warm the suspensions.

The superoxide anion radical was detected employing PBN as a spin trap in presence of DDC to inhibit SOD in the isolation medium. EPR spectra of the PBN-superoxide radical adduct were recorded on a VARIAN E-104 EPR spectrometer equipped with TM_{110} cavity. Instrument settings employed were: Scan range- 100G, Field set-3237 G, Temperature-27°C, Time constant- 0.5 sec, Scan time - 8 min, modulation frequency - 100 kHz, microwave power- 5mW, microwave frequency 9.01 Ghz, receiver gain- $2.5\times10^4\times10$ (unless other wise stated). The EPR absorption line intensities of the id field lines were calculated employing the equation $I=kw^2h$ (where $k=6.51\times10^{-10}$, a line shape constant; w= line width; h=line height; I - is the integrated intensity of the first derivative signal (28), which served as a measure to compare the quantity of superoxide radical generated (1, 28, 29).

Assay of superoxide dismutase activity in the thyroid gland of pigeons. Preweighed thyroid tissue was placed in 4 ml chilled tris-HCL buffer (50mM, pH-8.2) containing 1mM EDTA and homogenized at 4°C at a speed of 13,000 rpm (2cycles, 30 seconds each) using a Polytron homogenizer with PT-10 accessory. The homogenates were treated with 1 ml of 1% Triton X-100 so that the final concentration of the detergent was 0.2%. After treatment for 20 minutes, the suspensions were centrifuged at a speed of 15,000rpm at 4°C using a Sorvall OTD 65B ultracentrifuge and a T865.1 fixed angle rotor. The pellets were discarded while the supernatants were assayed for Sod activity by the method of Marklund and Marklund, 1974 (30) using the ability of the enzyme to inhibit autoxidation of pyrogallol, the enzyme kinetics was carried on a SLM AMINCO DW-2000 UV-VIS spectrophotometer. All calculations were made as per mg fresh weight.

Data analysis. Statistical analysis were made using introductory statistics software package (ISSP), Version 1.0 (31). The degree of variance of the observations obtained was tested by subjecting them to a one way ANOVA, where the results of each group is compared with that of the preceding group. The Pearson Correlation coefficient 'r' was calculated between the levels of SOD and $O_2^{\cdot-}$ using the above mentioned program.

RESULTS AND DISCUSSION

The EPR spectra of the trapped species in our study set up are presented in Figure 1. The presence of $O_2^{\cdot-}$ in the thyroid gland was confirmed by comparing the absorption line characteristics of PBN-$O_2^{\cdot-}$ adduct obtained when $O_2^{\cdot-}$ was generated artificially using a pyrogallol autoxidation system (Figure 1, G). The PBN-$O_2^{\cdot-}$ adduct is further verified by its characteristic nitrogen hyperfine splitting of a^N=14.81 G (16). The intensities of the mid field line indicated by solid arrows were calculated and served as a semiquantitative measure of the amount of $O_2^{\cdot-}$ present in a given system. The electron spin resonance signals gathered from the thyroid glands of control birds did not show detectable traces of $O_2^{\cdot-}$ radical-PBN adduct (Figure 1, F). The administration of TSH resulted in an immediate burst in $O_2^{\cdot-}$ radical anion noticeable 20 minutes after injection (Figure 1, E). The level of superoxide detected at 20 minutes after TSH injection appeared to be the peak value, which declined toward 40 minutes (Figure 1, D). At 60 minutes after thyrotrophin administration, there was a highly significant decline ($p<0.001$) in the levels of superoxide radical (Figure 1, C), which further decreased after 80 minutes (Figure 1, B) and 120 minutes (Figure 1, A) to a level not measurable by ESR spectroscopy.

Figure 2 also shows the induction and changes in SOD activity after thyrotrophin treatment during different time period. Even before the external administration of TSH, the thyroid gland exhibited a fairly good SOD activity. But, after the administration of 20 μg TSH, there was an immediate rise in SOD activity in the thyroid ($p<0.01$) noticeable at 20 minutes after injection and reaches a peak ($p<0.01$) in 60 minutes of TSH injection. When compared with the levels at 60 minute time point, there was a marked decrease ($p<0.001$) in SOD levels from 180 minutes onwards reaching a level comparable to the control value.

Changes in serum concentrations of T_4 and T_3 in re-

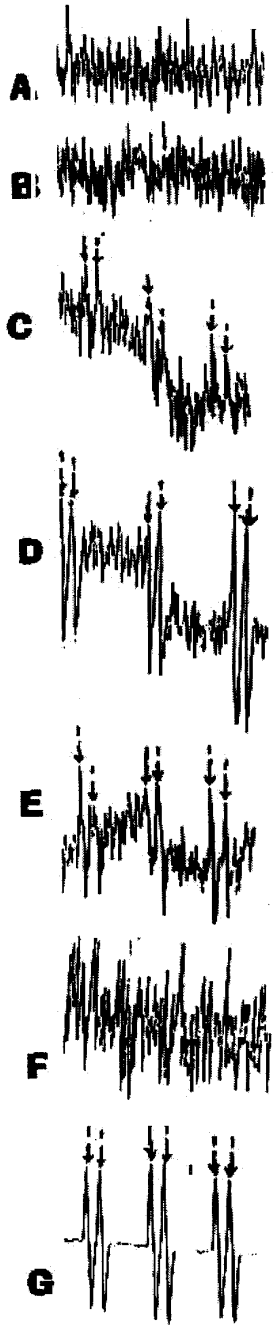

FIG. 1. EPR spectra of superoxide radicals (trapped as $O_2^{\cdot-}$-PBN adduct) generated by avian thyroid after different time intervals (F-0 minute control, E-20 min, D-40 min, C-60 min, B-80 min, A-120 min), under the influence of TSH. Reference spectrum of $O_2^{\cdot-}$-PBN adduct (G) was using a pyrogallol autoxidation system. Intensities of the low-field absorption lines (indicated by solid arrows) served as a comparative measure for the quantity of the adduct formed.

sponse to a single intraperitoneal injection of TSH are depicted in Figure 3. Administration of TSH significantly ($p<0.05$) elevated serum concentrations of T_4, but not T_3. TSH injection also caused progressive in-

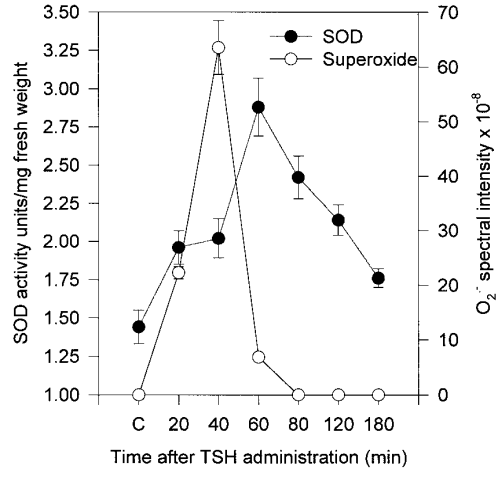

FIG. 2. Effect of TSH on the production of $O_2^{\cdot-}$ anion radical and SOD activity in the thyroid gland of pigeon. Results are mean±s.e.m. of three replicates.

crease in circulating T_4 level, which reached a plateau between 60 and 180 minutes after hormone treatment.

Thus, this is the first report concerning the response of thyroidal $O_2^{\cdot-}$/SOD system to thyrotrophin. The data presented here clearly suggest that TSH has a modulating effect on thyroidal superoxide radical and superoxide dismutase system during its tropic action in intrathyroidal phase of iodine metabolism. Our work provides an experimental evidence for the induction of SOD by TSH in the thyroid and also points to a well timed relationship of SOD induction to thyroid hormonogenesis. Since our results demonstrate a peak in SOD activity 60 min after TSH administration which

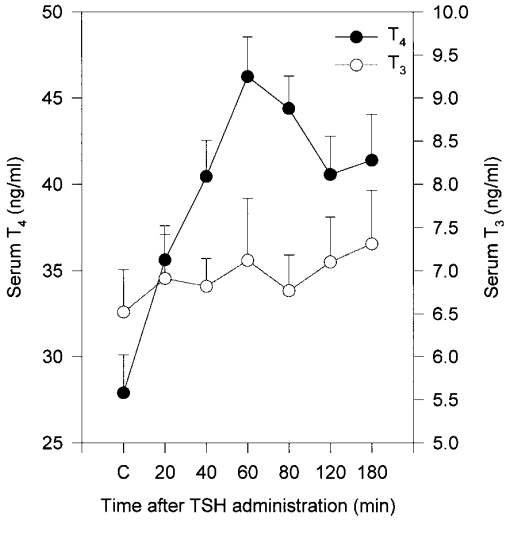

FIG. 3. Levels of serum T_4 and T_3 concentrations at different time intervals after TSH administration in adult male pigeon. Data represented here are mean values±s.e.m.

correlates very well with the peak level of T_4 between 60 and 180 min after thyrotropin administration ensuring sufficient levels of H_2O_2 for thyroidal iodide peroxidase during thyroid hormone biosynthesis. TSH never increased T_3 although T_4 is consistently elevated. In birds, the thyroid secretion consists of almost 100% of thyroxine (T_4) (32, 33). The type-1 iodothyronine deiodinase (ID-1) converts the prohormone T_4 by 5′-monodeiodination (5′-D activity) to bioactivate T_3 in peripheral tissues mainly liver and kidney (3, 34-38). Our results showing significant increase in serum concentration of T_4, but not T_3, confirms the previous observation that in birds TSH is mainly thyrotrophic having no influence on the peripheral conversion of T_4 in to T_3 by stimulating the 5′-D activity (39).

Maintenance of a fairly high SOD activity in control birds in this experimental study defines thyroidal SOD as a constitutive enzyme. Constitutive expression of SOD gene locus in other tissues has been already reported earlier (40, 41). Although SOD is a constitutive enzyme, its levels could be modulated by hyperbaric oxygen levels, oxidative conditions (40) and by hormonal influence (29). Hormonal induction of SOD in the ovary by LH and its complete blockade by anti-LH serum have been reported in the rat (29). It was postulated that H_2O_2 formed by enzymatic $O_2^{\cdot-}$ dismutation drives the peroxidase-ascorbate system responsible for the production of progesterone. Our finding of TSH induction of avian thyroidal SOD seems to explain that the primary effect of TSH in the thyroid gland is to activate $O_2^{\cdot-}$/SOD system at a time prior to peroxidase induction to produce enough H_2O_2 for a well-timed peroxidase dependent organification of thyroidal iodine. Thus, this study reveals a novel and fascinating mechanism of thyrotropic regulation of the pituitary gland in avian thyroid.

EPR signals of $O_2^{\cdot-}$-PBN adduct were extremely weak in the thyroid follicular cell preparations from control birds. This finding justifies the thyroidal SOD creating a potential sink for the toxic superoxide radicals. In this way, SOD serves as a strategic antioxidant enzyme to protect the thyroidal cells against oxygen toxicity and secondly to provide H_2O_2 for thyroid hormonogenesis. Our results contribute to think that $O_2^{\cdot-}$ radical is essential for many normal biological processes. In the present communication, we assign a role for $O_2^{\cdot-}$ in iodine metabolism by providing H_2O_2, a substrate for thyroidal iodide peroxidase. Recent research reports from Laloraya's lab in Indore have assigned numerous beneficial actions to $O_2^{\cdot-}$ radical and SOD system in mammalian and avian physiology (1, 3, 4, 29, 42-46). Furthermore, free radicals have been shown to be essential for many normal biological processes. They are involved in cycloxygenase and lipoxygenase action in eicosanoid metabolism, during the conversion of prostaglandin G_2 to prostaglandin H_2 and hydroperoxy eicosatetraenoic acid to the hydroxy derivatives; they are intermediates/products in enzyme catalyzed reactions, e.g., the formation of tyrosyl radical in the mechanism of action of ribonucleotide reductase, they are regulatory molecules in biochemical processes, e.g., nitric oxide (47, 48).

Under the influence of exogenous TSH, the burst of $O_2^{\cdot-}$ radical after 20 minutes and its complete scavenging after 60 minutes throw light on to the thyrotropin influenced change in thyroidal oxygen status and an adaptive induction of SOD. Immediately after injection, TSH increases thyroidal growth and general metabolic activities including glucose uptake, oxygen consumption and NADPH oxidation (2, 6, 49). NADPH oxidase, which generates $O_2^{\cdot-}$ radical in the thyroid is shown to be increased during thyroidal oxygen burst (2). Thus, the generation of $O_2^{\cdot-}$ radicals in our observation may be due to TSH induced activation of NADPH oxidase activity under TSH mediated increase in oxygen consumption rate of the thyroid gland. The complete scavenging of $O_2^{\cdot-}$ in a very short period is due to an increased level of SOD under TSH influence. SOD level reached its peak only after 60 minutes of TSH treatment, beyond which appears a gradual decline of $O_2^{\cdot-}$ radical. An inverse correlation between SOD and $O_2^{\cdot-}$ radical is clearly evident ($r=-0.78$) (Figure 2). It has been already reported that increasing an organism's exposure to dioxygen induces an increase in the level of SOD activity and there is a correlation between the cell's normal exposure to dioxygen and its SOD level (50). Thus, avian thyroid avoids glandular $O_2^{\cdot-}$ radical by an increased activity of SOD. The thyrotropic induction of thyroidal SOD protects the endocrine thyroid from the degenerative influence of $O_2^{\cdot-}$ radical. Suguwara et al (51) found a deficiency of cytosolic SOD in the thyroid tissue of patients with endemic goitre and suggested the decreased enzyme activity causes degeneration of the tissues, presumably due to prolonged exposure to oxygen free radicals.

It is concluded that avian thyroidal $O_2^{\cdot-}$/SOD system is under trophic control of the pituitary gland and TSH induces SOD activity profile to drive H_2O_2 dependent thyroid hormone biosynthesis. A probable clinical significance of the present investigation is that thyroidal SOD also acts as a potential free radical sink against toxic paramagnetic $O_2^{\cdot-}$ free radical and any deficiency of this antioxidant enzyme may lead to toxic effects under $O_2^{\cdot-}$-mediated oxidative stress, which may in turn affect normal growth, metabolism and function of the thyroid gland.

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